Lung Collagen Metabolism and the Clinical Course of Hypersensitivity Pneumonitis*

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We studied lung collagen metabolism in 18 patients with hypersensitivity pneumonitis to determine if changes at this level could explain the different clinical courses followed by these patients. Collagen concentration, biosynthesis and degradation were measured in lung tissue samples obtained before treatment. Four patients healed, eight improved and six did not improve or worsened. All patients who healed showed an important increase in collagenolysis; patients who improved had normal or high values, but significantly less than those obtained in patients who healed. Finally, five out of the six patients who did not improve or worsened had a significant decrease in degradation. These findings support the notion that a diminution of local collagenolysis may play a role in the progression to fibrosis in some patients with hypersensitivity pneumonitis and can also be a useful tool to predict the prognosis of this disease.

Hypersensitivity pneumonitis (HP) represents a group of lung diseases provoked by the inhalation of a variety of organic particles and characterized by a diffuse and predominantly mononuclear inflammation of the lower respiratory tract. Although the mechanisms involved in its pathogenesis are not completely understood, most authors agree that this disorder is triggered by a complex sequence of events, mainly mediated by a T-cell hyperreactivity.

Once considered a benign disease, that is, easily reversible by avoiding the etiologic agent and applying steroid therapy, it has currently become evident that some patients may follow a chronic course and continue to be symptomatic in spite of treatment. Moreover, some of them may evolve progressively to interstitial lung fibrosis.

Unfortunately, almost all the experimental and human studies have been focused on the pulmonary damage mechanisms, both immunologic and nonimmunologic, but few attempts have been made to determine why some patients heal, others remain with clinical and/or functional abnormalities and still others progress to fibrosis. Obviously, the clinical course of the disease may depend on a complex series of interrelated factors such as genetic predisposition, intensity and duration of exposure, evolution time before the onset of therapy, but other mechanisms may play a role. Disturbances in the appropriate control of collagen metabolism may be one of them.

In this context, we currently know that lung collagen turnover is an active and dynamic process, and changes in either synthesis or degradation could play an important role in the regulation of the deposit and amount of interstitial collagens in different lung diseases.14

Thus, we have recently demonstrated that patients with idiopathic pulmonary fibrosis (IPF) present a noteworthy decrease in the lung collagenolytic activity without an increase in collagen biosynthesis.15 However, these patients were studied in an advanced stage of the disorder when there was a clear predominance of the fibrosis over inflammation.

On the other hand, Laurent and McAnulty have reported a bleomycin-induced pulmonary fibrosis in rabbits that changes in anabolism and catabolism of this protein are early events in the development of fibrosis. Six days after drug administration, there was a significant increase in collagen synthesis and a decrease in the degradation of newly synthesized collagen. Similar results have been reported in other animal models of interstitial lung diseases (ILD).17

An indirect proof that a disarrangement of collagen turnover may play a role in the development of fibrosis in human ILD, including HP, is the finding by Anttinen et al who reported that galactosylhydroxylsyl glycosyltransferase, an enzyme which catalyzes collagen biosynthesis, is increased in patients with pulmonary fibrosis and in some with alveolitis who often develop fibrosis later. Nevertheless, direct studies on lung tissue of patients suffering HP are scanty.

With these precedents, the aim of our study was to analyze the behavior of lung collagen metabolism in patients with HP induced by avian antigen, with the

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purpose of knowing if some changes at this level could explain, at least partially, the different clinical courses followed by these patients.

PATIENTS AND METHODS

Study Population

We studied 18 nonsmoking female patients, aged 18 to 62 years old (mean age: 37.4 years), who had a history of close exposure to avian antigen. The diagnosis was suspected because of the relationship between antigen exposure and the onset of the disease, and by the presence of clinical, radiologic and functional respiratory abnormalities suggestive of HP.23 The main features were progressive dyspnea, bilateral radiographic shadowing without hilar adenopathy, predominantly restrictive functional impairment and hypoxemia at rest which usually worsened with exercise. In addition, all of them had positive serum-specific precipitating antibodies to avian antigens determined by an enzyme-linked immunosorbent assay (ELISA).

The diagnosis was confirmed through an open lung biopsy, which was performed before giving any treatment. The morphologic findings were consistent with the diagnosis of HP described elsewhere.23 Briefly, the tissue samples showed a diffuse interstitial inflammation of mononuclear predominance, mainly lymphocytes, and frequently multinucleated giant cells in terminal and respiratory bronchioles as well as in the interalveolar walls. Foam macrophages were seen in the alveolar spaces, and small and loosely arranged granulomas were observed in interstitium.

Biopsy cultures were negative for bacteria, mycobacteria and fungi and no changes suggestive of another ILD were found. In 15 of the 18 patients, the lung tissue presented only inflammatory alterations. In the other three cases (patients 13, 14 and 16) a slight degree of fibrosis (less than 10 percent of the specimens) could be observed with the Masson's trichrome stain.

The average duration of symptoms at the time of lung biopsy ranged from three months to three years, with a mean of 13.6±2.6 months. Since in all patients breathlessness preceded the medical consultation, the onset of this symptom was taken as evidence for the clinical initiation of the disease.

Control Subjects

Seven control subjects (five women and two men) were selected from among a group of individuals who had lobectomy or wedge resection for removal of a primary lung tumor, but without any clinical, radiographic or functional evidence of ILD. Moreover, no histologic evidence of disease was found in the lung tissue specimens used for biochemical analyses. The ages of the control subjects ranged from 20 to 39 years, with a mean age of 39.4±12.2 years (Table 1).

Handling of Tissue Samples

Immediately after the biopsy, lung specimens were divided into four portions. One portion was fixed with buffered 10 percent formaldehdyde and afterward treated according to the conventional techniques for light microscopy. The other three portions were used for the biochemical analyses.

Collagen Concentration

Lung samples of about 100 mg (wet) were dried to constant weight, and three aliquots were hydrolyzed with 6N hydrochloride (HCl) for 24 hours at 100°C, filtered, dried, and resuspended in distilled water. Subsequently, hydroxyproline content was measured by a colorimetric assay.20 The amount of collagen in each aliquot was calculated by multiplying hydroxyproline content x 7.23, on the assumption that this residue constitutes about 14 percent of the total amount of amino acids in the alpha chain. Collagen concentration was expressed as milligrams per gram of dry weight.

Collagen Biosynthesis Assay

The method used is described elsewhere.25 Briefly, lung samples were divided in three portions weighing about 75 to 100 mg (wet). These samples were incubated separately in 4 ml of Dulbecco's modified Eagles medium containing 10 percent fetal calf serum, 50 µg/ml ascorbic acid, 70 µg/ml ferrous sulfate (the last two reagents were used as cofactors of proline hydroxylase), 200 U/ml penicillin, and 200 µg/ml streptomycin.

The cultures were equilibrated with 95 percent oxygen/5 percent carbon dioxide and incubated at 37°C in a shaking water bath. After one hour, the medium was replaced with 4 ml of fresh culture medium with the above constituents and also containing 30 µCi of [3H]-proline (L-[2,3-3H]-proline, 32.2 Ci/mmol, New England Nuclear, Boston, MA). Afterward, the cultures were incubated for four hours. At the end of the incubation period, the tissue samples were homogenized with a polytron tissue homogenizer (Brinkman Instruments, Westbury, NY) in 10 percent trichloroacetic acid (TCA), and washed three more times with 5 percent TCA. The TCA precipitable material was hydrolized for 24 h in 6N HCl at 100°C, filtered, evaporated, and dissolved in 2 ml of distilled water. The [3H]-hydroxyproline was separated according to the method of Rokind and Gonzalez26 and counted in a beta scintillation counter (Beckman LS-100C). The results were expressed as counts per minute of [3H]-hydroxyproline per milligram of incubated tissue per hour.

Collagen Degradation Assay

We used a modification of the method by Ryan and Woessner.21 Lung tissue samples of about 500 mg (wet) were homogenized and divided into six aliquots. Three of them were incubated in a metabolic shaker for 24 h at 37°C in the presence of 5.0 mM calcium chloride, 0.15M sodium chloride and 0.04M Tris buffer pH 7.4. The remaining three aliquots were incubated under the same conditions but 0.4M ethylenediaminetetraacetate (EDTA [collagenase inhibitor]) was added. Only EDTA-inhibitable degradation was considered as specific collagenolytic activity.

With the purpose of measuring only degraded collagen, the homogenates were centrifuged at 4°C for 30 min at 21,000 g, and the supernatant was filtered through a membrane with an exclusion limit of 100,000 daltons (Difco XM-100, Amicon Corporation, Lexington, MA). Thus, the fragments obtained were smaller than an alpha chain of collagen. Digestion was detected by the release of soluble hydroxyproline-containing material.

With the aim of controlling the ratio of active enzyme to substrate, the collagen content was quantified separately in each aliquot of the homogenate, and the collagenolytic activity was expressed as micrograms of collagen degraded per milligram of collagen incubated per hour. This assay measures collagen degradation as a whole, and no attempt was made to establish which particular collagen (type 1, type 3 or another) was more or less affected.

Follow-Up

Two weeks after the lung biopsy, the patients were conventionally treated with prednisone and then followed-up between one to three years. Prednisone was administered at a dose of 1 mg/kg for one month, followed by a gradual reduction of about 5 mg of the total dose each three or four weeks, until a maintenance dose of 10 to 15 mg/day was reached. If a patient was considered to be healed, this dose was sustained for another month, and then suspended. The patients who showed an objective improvement but did not heal continued with the maintenance dose. In the case of the patients who worsened, prednisone was newly increased, and maintained at 20 to 30 mg/day during the complete course of this study.

After the last study, patients were classified in three groups according to their clinical, radiologic and functional findings, as follows. Group 1 (n = 4): Healed, when there was complete disap-
pearance of dyspnea, and the functional respiratory tests and thorax x-ray film had reached normality. Group 2 (n = 8): Improved, when there was an objective and subjective amelioration of dyspnea, an increase of at least 15 percent in the restrictive functional alterations (vital capacity [VC] and/or total lung capacity [TLC] and/or compliance), an improvement of at least 7 mm Hg in the rest hypoxemia and a decrease of abnormal images in thorax x-ray films. Three of these parameters were considered as the minimal requirement to classify a patient as "improved." Group 3 (n = 6): Without improvement or worsened, when the clinical, radiologic and functional alterations remained without significant changes and/or when the patient showed an increase in dyspnea, in radiographic abnormalities and/or in the restrictive functional impairment and hypoxemia.

Statistical Analyses

For each variable studied (age, onset of symptoms, functional respiratory tests, collagen concentration, collagen synthesis and collagenolytic activity) the values were averaged and the standard deviation calculated. The significance of the differences between values obtained from the various groups was tested using the Student's t test. We also used the analysis of variance (ANOVA) for a one-way classification to test inter-group differences. Probabilities of 0.05 or less were considered significant. On the other hand, to establish the predictive value for collagenolytic activity, we calculated the sensitivity, specificity and efficiency according to Galen and Gambino.22 In addition, for this parameter we applied the receiver-operating characteristic (ROC) curve for each group. The inflexion point of the curve of each group provided the highest efficiency value. Afterward, we calculated the positive and the negative predictive value for each group.

RESULTS

All patients were symptomatic at the beginning of this study and their degree of breathlessness varied from moderate efforts to dyspnea at rest. Likewise, they presented a characteristic restrictive pattern with a variable decrease of TLC (average: 68.3 ± 11.7 percent, ranging from 46 to 86 percent), VC (average: 49.4 ± 13.9 percent, ranging from 20 to 67 percent) and compliance (average: 38.5 ± 10.7 percent, ranging from 22 to 55 percent). Some patients also showed a slight to moderate obstruction of peripheral airways. Finally, all of the patients had hypoxemia at rest average: 49.8 ± 6.8 mm Hg, ranging from 34 to 60 mm Hg (normal values for Mexico City at 2,240 m altitude: 67 ± 3 mm Hg) which worsened, in a lesser or greater degree, with exercise. However, none of these parameters recorded at the onset of therapy related with the future response to treatment. Thus, for example, patient 8 healed and case 16 improved, whereas patient 17 did not show any clinical or functional response to therapy (Tables 2 and 3) in spite of very similar severity in dyspnea and gas exchange abnormalities (arterial oxygen pressure at rest of 34, 44 and 40 mm Hg, respectively).

Table 1 summarizes the individual data obtained from control subjects, and Tables 2 and 3 show the patients' ages, the evolution time of the disease prior to the biopsy, the biochemical results and their relationship with the clinical course after one to three years of follow-up. The patients were separated according to their response to treatment into the three groups previously mentioned.

The four patients who healed had a mean age of 26 ± 7.8 years, which was significantly lower than the average age of the other two groups (p<0.05). In contrast, the evolution time of the disease before biopsy and the onset of therapy did not seem to influence the prognosis. In this sense, the four patients who healed and five of six patients who did not improve or who worsened showed an onset of the clinical disease of 12 months or less.

In relationship to the biochemical analyses, three patients (Nos. 13, 14 and 16) showed a slight increase of collagen concentration, which coincided with the morphologic observations of the lung. One of them improved and the other two did not. Nevertheless, average collagen concentration in the three groups was similar to that in control subjects, which was expected for the inflammatory phase of this disease and demonstrated in the histologic study of the lung samples.

Collagen biosynthesis data showed a broad dispersion. Eight patients presented an increase of ^H-hydroxyproline production in the short-term lung explant cultures; however, this modification did not bear any relationship to a bad clinical course since most of these patients healed or improved. Interestingly, in five of them the increase of collagen synthesis coincided with an elevation in the rate of collagen

### Table 1—Age, Sex and Biochemical Results in the Control Group

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Collagen Concentration (mg/g Dry Weight)</th>
<th>Collagen Synthesis (cpm [^H]-OH-Proline/mg/h)</th>
<th>Collagen Degradation, (μg Collagen Degraded/mg Collagen incubated/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>F</td>
<td>179</td>
<td>42.4</td>
<td>0.200</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>F</td>
<td>178</td>
<td>72.4</td>
<td>0.220</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>F</td>
<td>212</td>
<td>26.4</td>
<td>0.240</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
<td>F</td>
<td>202</td>
<td>24.0</td>
<td>0.300</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>M</td>
<td>196</td>
<td>45.9</td>
<td>0.254</td>
</tr>
<tr>
<td>6</td>
<td>58</td>
<td>F</td>
<td>144</td>
<td>40.0</td>
<td>0.286</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>M</td>
<td>185</td>
<td>42.0</td>
<td>0.250</td>
</tr>
<tr>
<td>x ± SD</td>
<td>39.4 ± 12.2</td>
<td>185 ± 22</td>
<td>42.3 ± 16.0</td>
<td>0.250 ± 0.035</td>
<td></td>
</tr>
</tbody>
</table>
degradation. On the other hand, only one case of the six who showed no improvement or worsened presented an increase of collagen synthesis, and in this patient collagenolysis was lower.

In comparison with biosynthesis, a different picture emerged from the analysis of the collagenolytic activity (Tables 2 and 3 and Fig 1). In this context, there were noteworthy differences between the three groups. Thus, all of the patients who healed showed a several-fold increase of collagenolysis, with an average of 0.877 ± 0.16 μg of collagen degraded per milligram of collagen incubated per hour (p<0.001 in relation to the control subjects). Patients who improved showed normal or high values, and their average was of 0.431 ± 0.242, which is higher than that in control subjects but significantly lower than the mean obtained for patients who healed (p<0.05). In contrast, five out of six patients who did not show improvement or worsened presented a decrease in collagenolytic activity of approximately one half to normal values (p<0.001).

In order to obtain a ratio between collagen synthesis and collagen degradation, we made uniform the expression of both parameters and formulated a metabolic index (synthesis/degradation). In this context, we found that whereas the average of control subjects was 1.0, group 1 showed a decrease in this index with an average of 0.5. On the other hand, group 2 presented an increase of this ratio with a mean of 1.49, due to an elevation of collagen production. Finally, group 3 showed the highest metabolic index, with an average of 2.17; however, the increase in this case was due to a decrement in collagenolytic activity.

When we applied the ROC curve to analyze the sensitivity, specificity, efficiency and predictive values of collagen degradation in relation to the prognosis, we observed that when the collagenolytic activity is higher than 0.6, the positive predictive value for healing is 80 percent. In contrast, when it is less than 0.15 the positive predictive value for not improving or worsening is 100 percent.

**DISCUSSION**

One of the most intriguing and less studied questions concerning HP is why, regardless of the type and time of exposure to an organic particle, the avoidance of...
patients who healed or improved and those who did not improve or worsened. Thus, whereas the former presented elevated rates of collagenolysis, the latter showed a noteworthy decrease in collagen breakdown. In this sense, we can hypothesize that an increase in local collagenolysis is a defensive mechanism to avoid excessive accumulation of collagen, and in this way, the development of fibrosis.

Interestingly, a better response was also seen in younger patients, similar to the finding by Turner-Warwick et al. in IPF, but the age of patients and control subjects did not show a direct relationship to lung collagenolysis results.

Our findings related to collagenolytic activity in HP agree with those reported for IPF in which all patients with fibrotic predominance showed decreased collagen degradation, and the same has been demonstrated in other fibrotic organs. Moreover, a decrease in collagenolysis seems to be an early phenomenon in experimental lung fibrosis.

Nevertheless, other authors have suggested that collagenase may play a role in the development, and not in the arrest of reversal, of the fibrotic process in some ILD. However, these studies have several potential methodologic drawbacks, for example, the use of bronchoalveolar lavage (BAL) as a source of collagenase, and soluble radioactively labelled collagen produced in vitro as substrate. In our opinion, this type of assay does not resemble the events occurring in the lung microenvironment, where collagen in the extracellular space exists in an insoluble form and in a complex state of aggregation with other interstitial macromolecules, and therefore, is probably less susceptible to the action of the enzyme. In this sense, our assay is a closer approach to the parenchymal situation.

In support of this point of view, Christner et al., using a similar technique to the studies mentioned before, also have found collagenase activity in BAL of patients with adult respiratory distress syndrome (ARDS), which may evolve to fibrosis, but this finding seemed associated with the lung inflammatory response rather than with the development of fibrosis. Thus, the enzyme was present both in ARDS survivors and nonsurvivors, and the authors postulate that the increase in degradation may be necessary in order to return the lung to its baseline physiologic state.

On the other hand, our findings may be interpreted from two interrelated points of view.

One of them is the role of the triggering of modifications in collagen turnover regarding the progression of HP to fibrosis.

In this study, no histologic evidence was obtained to prove that the patients who did not improve or worsened developed interstitial lung fibrosis because, for ethical reasons, a second biopsy was not performed.
However, in a previous study we found that in the chronic and advanced form of HP induced by avian antigen there is an increase in collagen content and morphologic evidence of lung fibrosis. In the current study the clinical, radiologic and functional abnormalities strongly suggested that the same situation occurred in these patients.

We believe that HP, as many other ILD, may progress in some cases to fibrosis, and the linkage between inflammation and fibrosis will depend on an imbalance of the normal homeostasis of collagens in which synthesis exceeds breakdown, resulting in an excessive accumulation of this protein. Interestingly, the findings of this study suggest that changes in collagen turnover almost always precede collagen deposit, measured by both biochemical and morphologic analyses. In addition, our results support that collagen degradation is more important than collagen synthesis in the regulation of lung collagen mass. Thus, the patients who showed elevated rates of collagen synthesis improved or healed almost always whenever there was a concomitant increase in the local collagenolytic activity. Moreover, although group 2 presented an increased ratio between synthesis and degradation, this was due to an elevation of collagen production, and these patients improved. In contrast, in group 3 there was a higher metabolic index, due almost exclusively to a decrease in collagenolysis, and these patients showed no improvement or worsened. Therefore, the increase in collagen synthesis may be a transient stage which could be controlled if an appropriate state of collagenolysis is subsequently developed. This line of thought agrees with our results, which show that patients with lower rates of degrada-

![Figure 2](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21581/) Figure 2. Hypothetical view of collagen homeostasis during evolution of human interstitial lung disease which evolves to fibrosis. Regarding collagen degradation, A represents an increase as a part of the overall inflammatory response and B, a defensive mechanism against the abnormal accumulation of interstitial collagens. In the fibrotic phase, the ratio between synthesis and collagenolysis is always higher than normal, regardless of their individual values.

dition did not improve or worsened in spite of normal values of synthesis, and with our previous report on IPF in which a very similar alteration was found. A hypothetical view concerning the homeostasis of collagen in relation to the evolution of interstitial lung diseases which evolve to fibrosis is shown in Figure 2.

However, collagenolysis is a very complex mechanism which involves several steps, with intracellular and extracellular pathways, and the regulation and relative importance of each of them is just beginning to be analyzed, so that more time and more detailed studies are needed to allow for definitive conclusions on its precise role in the pathogenesis of lung fibrosis, including that secondary to HP.

In addition, several classes of collagenases have been described, secreted by different types of cells, with varying specificities for interstitial and noninterstitial collagens, and whereas the action and degree of substrate specificity of the former are relatively well-known, in the latter they are still unclear. Furthermore, the cell or cells responsible for the secretion of different types of collagenases, and the stimuli which trigger their production in the lung inflammatory disorders are unknown.

On the other hand, our results also suggest that the analysis of collagenolytic activity may be a useful tool to predict the clinical course and the prognosis of HP. Thus, values higher than 0.60 result in a positive predictive value of 80 percent for healing, with a sensitivity of 100 percent and specificity of 93 percent. In contrast, values lower than 0.15 present a positive predictive value of 100 percent with a sensitivity of 83 percent and specificity of 100 percent for not improving or worsening. The negative predictive values are
similar.

Since lung biopsy is usually performed before treatment, the measurement of this biochemical parameter, along with others such as procollagen type 3 peptide, may allow clinicians to have a better approach to prognosis. Perhaps in the future these patients may be treated with some of the antifibrotic drugs currently under study besides steroids.

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